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Continuous production of class III plant peroxidase by the immobilized callus cultures of *Tecoma Stan* Var. *Angustata* using a packed bed reactor

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Abstract
A new system to continuously produce class III plant peroxidase enzyme by the immobilized callus cultures of *Tecoma Stan* var. *angustata* using a Packed Bed Reactor is described. Batch cultures were used for the partial characterization and optimization of the enzyme. A maximum productivity of 2478 X 10^{4} U l^{-1} h^{-1} was obtained at a residence time of 15 X 10^{-3} h using full-strength Murashige and Skoog's (MS) medium. At optimum conditions of flow rate (0.3 ml min^{-1}), sucrose concentration (20 g l^{-1}), pH (5.0) and temperature (50 °C), the half-life of the enzyme production reached only on the 50th day when fed with half-strength MS medium. Metal ions (1mM concentration) such as Fe^{2+}, Ca^{2+}, Cu^{2+} and Na^{+}, when provided separately along with the culture medium rapidly increased the enzyme activity on the 50th day of its production.

Keywords: Calcium alginate, callus, continuous production, peroxidase, *Tecoma Stan*

Introduction

Peroxidase enzyme has got wide applications in bioremediation and waste water treatment (Singh *et al*., 2023) [22], for the biotransformation of various drugs and chemicals (Egorov *et al.* 2000) [8], in enzymatic determination of phenols (Bagirova *et al.* 2001) [2], diagnostics and biosensors (Bilal *et al.,* 2023) [4], in the construction of time temperature indicator (Rani ND & Abraham TE, 2006) [17], in dye degradation (Shaffique *et al*. 2002, Mohan *et al*. 2005) [13, 21] etc.. This enzyme shows high thermal stability and substrate specificity. (Rani ND and Abraham TE 2006) [17]. Plant Tissue culture has been used for the micro propagation, callus proliferation, secondary metabolite production etc. (Rani ND and Nair GM, 2006) [19]. Although there are some reports on the continuous production of LiP (Mtui & Nakamura 2002, Couto *et al.* 2002) [15] and MnP (Moriera *et al.* 1997) using micro-organisms, plant callus cultures have been given little attention for continuous enzyme production. Major limitation of plant peroxidase is the low yield and high cost of production compared to the microbial enzymes. Reduction in enzyme cost can be achieved through continuously reusing the enzyme after immobilizing on various supports (Duran and Esposito, 2000) [7] and by decreasing the purification cost. In our study, callus cultures of *Tecoma stans* var. *angustata* (Family–Bignoniaceae) were immobilized in calcium alginate and were packed in a Packed Bed Reactor (PBR) for the continuous production of the enzyme. Plant callus cultures were used for the study as it offers a much purer form of enzyme compared to microbial enzymes. Entrapment is the most widely used technique for immobilization of whole cells and calcium alginate was used as the matrix as it is non-toxic and mild towards the biological materials (Kourkoutas *et al*. 2004) [12]. Optimization of enzyme production based on medium concentration, flow rate, sucrose concentration and effect of metal ionic inducers was also studied. Parameters like flow rate, dilution rate, residence time and productivity were calculated in addition to V_{max} and K_m for the catalysis of substrates, ABTS and H_2O_2.

Materials and methods

Tissue Culture and Callus Initiation

Leaf segments with midrib (7 X 10 mm) were washed well in 10% (v/v) labolene (a neutral surfactant) for 10 minutes followed by a treatment in 0.1% (w/v) mercuric chloride for 5 minutes and inoculated in the media containing different concentrations of the plant growth regulators (PGRs) like 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with benzyladenine (BA) on full-strength MS medium (Murashige and Scoog 1962) [16]. 30 g sucrose l^{-1} was used as the carbon source and 8g agar l^{-1} as inert solidifying agent, and incubated at 25 ± 2 °C under a photo period of 12/12 h. Durations for optimum biomass growth and enzyme activity were also detected. All the experiments were repeated thrice with 10 replicates each.

Immobilization technique

4g sodium alginate l^{-1} and 10 g callus cells l^{-1} in distilled water were mixed well using magnetic stirrer. The mixture was extruded through a needle into 0.06 M $CaCl_2$ solution to yield calcium alginate beads of 3 to 4 mm diameter, that were kept in curing solution of 0.025 M $CaCl_2$ in a refrigerator. Before use, the beads were washed well with sterile water.

Assay procedure for immobilized peroxidase enzyme

In order to find out the enzyme activity of the immobilized callus, the beads were given an incubation period of 10 minutes in the appropriate buffer prior to it being introduced into the cuvette at definite intervals of time and the increase in absorbance per minute was monitored. Activity retained by the immobilized enzyme was calculated using the formula

$$\text{Activity Retention (\%)} = \frac{\text{Immobilized Enzyme Activity}}{\text{Initial Activity of the enzyme in callus}} \times 100$$

Peroxidase activity was assayed by the method of Bergmeyer using ABTS as substrate (Bergmeyer 1983) [3]. The experiment was performed in two steps. The optimization of pH, temperature and the effect of metal ions

were standardized in the first step using the beads as batch cultures and the second step involved the construction of a Packed Bed Reactor (PBR). Optimum productivity of the bioreactor was studied by varying the flow rate, medium concentration and sugar concentration. Metal ionic inducers were used for increasing the enzyme production from the bioreactor after it reached the half-life (50 days).

Optimization and kinetic studies as batch cultures

Optimization of pH and temperature was done by the batch culture experiments. pH optimum was determined by incubating the enzyme for 20 minutes in appropriate buffer from pH 3 to 8 in 0.05 M buffer (3 to 4–citrate phosphate buffer; 4.5 to 5.5–acetate buffer; 6 to 8–phosphate buffer). To find out the optimum temperature, the enzymes were subjected to different temperatures ranging from 20–90 ºC, for 25 minutes in 0.05 M acetate buffer, pH 5, the enzyme samples were quickly cooled on ice and the activity was studied. The substrate used was 0.36 mM ABTS in all these studies (H_2O_2–5 mM).

Effect of different concentrations (0.35–1.5mM) of the reducing substrate, ABTS on the reaction rate of the enzyme was studied in 0.05 M sodium acetate buffer pH 5 at constant H_2O_2 (5 mM) concentration. The same was done with H_2O_2 (0.1–6 mM), keeping ABTS concentration constant (1.3 mM). Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were studied using Sigma plot (7.01 version) 2001 software. All the experiments were repeated thrice with three replicates each.

Effect of metal ions on enzyme activity

In order to find out the influence of different metal ions like Cu^{2+}, Fe^{2+}, Ca^{2+}, Na^{+} and Zn^{2+} on enzyme production, 1mM concentration of each metal ion (as compound) was given in the medium separately and the activity was studied after an incubation period of 25 minutes. Mean of five independent experiments using three replicates each was taken.

Construction of a PBR and its operational parameters

The bioreactor consisted of a jacketed glass column of 14 cm height and 3 cm internal diameter. The temperature was maintained at 50 °C by the circulation of thermostated water. The immobilized beads were packed in the column as shown in figure 2 from which the bioreactor parameters like void volume (V_0), dilution rate (D), residence time (R) and productivity were calculated. The feeding medium was pumped to the bioreactor in a continuous up-flow mode at different flow rates. The samples from the reactor were collected every day, after attaining a steady state and assayed for enzyme activity. All the experiments were done at sterile conditions.

Optimization studies of the bioreactor

The reactor was fed with full-strength MS medium with 30 g sucrose l^{-1} at different flow rates of 0.3, 0.5 and 1 ml min^{-1}. At the optimum flow rate, optimization of the medium concentration was done by feeding the reactor with full, 1/2 and 1/4 strength of nutrients. Different concentrations (5 to 30 g l^{-1}) of sucrose was given with 1/2 strength MS (at 0.3 ml min^{-1}) to study the optimum concentration of sucrose on continuous enzyme production.

Results and Discussion

Enzymes are usually extracted from the microbial cultures and from the plant biomass, the recovery and purification becomes expensive and technically challenging, requiring multiple separation steps such as ammonium sulphate precipitation, dialysis, lyophilization, ion exchange chromatography, size exclusion chromatography etc. whereas tissue culture offers a simplified and much cheaper method for the product recovery, eliminating the need to destroy the cells. Peroxidase obtained from the callus cultures of the plant, *Tecoma stans* showed a maximum activity of 259 U g^{-1} which is ten times more than that obtained directly from leaves (23 U g^{-1}). Peroxidase activity as well as callus proliferation and type of callus varied depending on the type and concentration of hormones used (Data not shown). Callus induced by BA and 2,4-D together was friable and produced a maximum of about 9 g dry weight biomass l^{-1}. Optimum biomass growth as well as enzyme activity were obtained on the 20th day of inoculation by the combined effect of 1.0 mg BA l^{-1} and 0.05 mg 2,4-D l^{-1} (Figure 1). Beyond this period, reduction in callus proliferation occurred due to the depletion of nutrients that was followed by a gradual decline in enzyme production. Induction of peroxidase activity by Cytokinins like BA has already been reported by Kapchina-Toteva & Yakimova (1997) [11] during their *in vitro* studies on apical dominance. Callus showing maximum peroxidase activity of 259 U g^{-1} obtained by the aforementioned hormone combination was used for immobilization in calcium alginate. The immobilized callus showed an activity retention of 86% and no reduction in activity was noticed even when it was reused for 6 cycles as batch cultures. In the first part of our experiment using batch cultures, we found out the optimum pH and temperature for the enzyme production to be 5 and 50 °C respectively. During the continuous production, these conditions were kept constant. The enzyme was also found to be activated by some metal ions supplied at 1 mM concentration and the relative activity percentages are shown in Table 1. The increase in peroxidase activity by metal ions like Cu^{2+}, Fe^{2+}, Zn^{2+} can be due to the formation of new isozymes in response to metal stress as is reported previously in whole plants and detached leaves of pepper and rice respectively (Diaz *et al.* 2001 and Fang & Kao 2000) [6, 9]. Increase in enzyme activity with Fe^{2+} can also be due to the presence of haem at the catalytic site of peroxidase. Effect of Ca^{2+} can be also attributed to the presence of 2 calcium ions in the enzyme molecule thereby enhancing the activity. These optimization studies performed in batch cultures were helpful for the bioreactor studies. The bioreactor fed with full-strength MS medium gave a maximum productivity of 2478 X 10^4 U l^{-1} h^{-1} (403 U ml^{-1}) at a flow rate of 0.3 ml min^{-1}, three times to that at 1 ml min^{-1}. The strength of the medium was reduced in order to reduce the cost of enzyme production and half strength MS medium was found to be better and more economic as it retained almost the same activity as with full strength at 0.3 ml min^{-1} (Table 2). The optimum sucrose concentration for enzyme production was 20 g l^{-1} (Figure 3). The activity became half only after a period of 50 days with no deterioration of the immobilized beads when the flow rate was kept at 0.3 ml min^{-1}. Flow rates below 0.3 ml $^{-1}$ was not tried as it required more residence time and higher flow rates was also not suitable as it lead to wastage of nutrients. The reactor was fed with 1mM concentration of metal ions like Cu^{2+}, Fe^{2+}, Ca^{2+}, Na^{+} and Zn^{2+} after the activity reached the half-life. Maximum increase in activity was observed with Fe^{2+} followed by Ca^{2+}. Nearly 25%

activity was increased by the influence of Cu^{2+} and Na^{+} and the results were in concordance with the batch culture experiments (Figure 4). Enzyme showed a K_m and V_{max} of 0.76 mM and 117.6 µmol min^{-1} mg^{-1} respectively, using ABTS as substrate. (Figures 5).

Although the bioreactors using microbial cultures require less initial cost, tedious purification steps due to the turbidity of the crude enzyme and wash out of microbes make them inferior to the plant callus cultures. Though there are a large number of reports on the characterization and optimization of peroxidase from the plant callus cultures (Castillo *et al.,* 2023). as far as we know, there is no other report on the continuous production of class III peroxidase using plant callus cultures, may be due to the high cost of production. Table 3 shows a comparison of different systems for continuous production of different peroxidases using bioreactors. Further reduction in the cost of bioreactor construction can be achieved by using callus cultures initiated by less costly plant growth regulators and also using more inducers alone or in combination.

Scale up studies can be very much promising for various applications like sparkling of wine, colour removal of molasses and dye degradation.

Conclusions

Continuous production of class III peroxidase enzyme from callus cultures of *Tecoma Stan* var. *angustata* was investigated using a jacketed Packed Bed Reactor. 0.018 l h^{-1} (0.3 ml min^{-1}) was found to be the optimum flow rate for enzyme productivity. Half-strength MS medium yielded 2418 X 10^4 U l^{-1} h^{-1} enzyme at optimum conditions. Partial characterization of the enzyme as well as V_{max} and K_m values for the catalysis of ABTS and H_2O_2 was also studied. Feeding of the medium with metal ions after reaching the half-life gave very interesting results. It is for the first time that continuous production of class III peroxidae enzyme is being performed using immobilized callus cultures using a PBR and our results provide the basic data for the large-scale continuous production of this important enzyme for various applications.

Table 1: Effect of metal ions on enzyme activity in batch cultures of *Tecoma Stan*

No	a Reagent (1 mM)	Relative activity (%)
1	None	b100
2	$CuSO_4$	126
3	$FeSO_4$	189
4	$CaCl_2$	151
5	$HgCl_2$	96
6	NaCl	129
7	$ZnSO_4$	112

(pH 5, 50 °C, 1.3 mM ABTS, 1 mM H_2O_2)

[a] 1 mM metal ion (as compound)

[b] Activity of 120 U ml^{-1} in the absence of metal ions was taken as 100%

Table 2: Parameters of continuous production of peroxidase. The dilution rate (h^{-1}), D was calculated using the formula, $D = F/V_0$ where F = flow rate (h^{-1}) and V_0 is the void volume (l). The Residence time (h), R = 1/D. Productivity (U l^{-1} h^{-1}) = Enzyme activity U l^{-1} X D

Medium	Flow rate (l h^{-1})	Dilution rate (h^{-1})	Residence time (h)	Productivity (U l^{-1} h^{-1})
Full MS	0.06	214	4.7 × 10^{-3}	876 × 10^4
	0.03	107	9.3 × 10^{-3}	2268 × 10^4
	0.018	64	15 × 10^{-3}	2478 × 10^4
1/2 MS	0.018	64	15 × 10^{-3}	2418 × 10^4
1/4 MS	0.018	64	15 × 10^{-3}	1203 × 10^4

Table 3: Comparison of different systems for continuous productions of peroxidase enzymes in bioreactors

No	Biological system	Bioreactor	Operation time (d)	Maximum enzyme activity (U ml^{-1})	Reference
1	*Phanerochaete chrysosporium*	Column reactor	120	Lignin peroxidase (0.576)	Rogalsky et al. (1992)
2	*Phanerochaete chrysosporium*	Packed bed	140	Manganese peroxidase (0.3)	Moreira et al. (1997)
3	*Bjerkandera adusta*	Jar fermenter	60	Lignin peroxidase (220); Manganese peroxidase (150); Laccase (50)	Mtui & Nakamura (2002)
4	*Phanerochaete chrysosporium*	Fixed bed	26	Lignin peroxidase (0.7)	Couto et al. (2002)
5	*Tecoma stans var. angustata*	Packed bed reactor	50	Class III plant peroxidase (403)	Present study

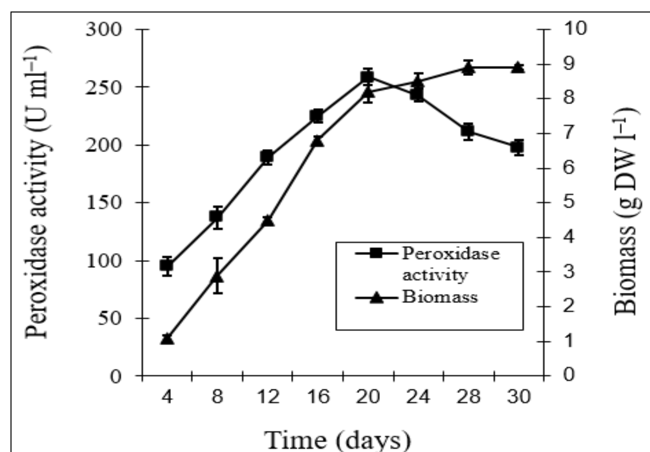

Fig 1: Time course on peroxidase activity and biomass growth of *Tecoma Stan* callus cultures

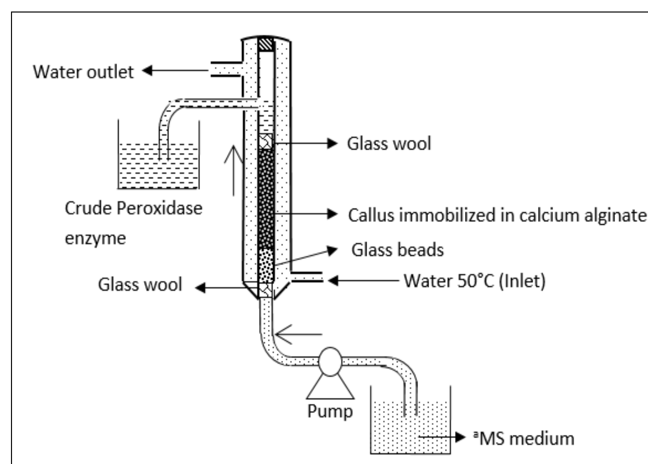

Fig 2: Diagrammatic sketch of the packed bed reactor

To calculate the dry weight, callus cultures were collected by vacuum filtration. After washing three times with deionized water, fresh weights were determined. They were then dried for 24 h at 80°C in an oven for determination of dry weight.

The bioreactor is made up of glass column with dimensions of 14 cm height and 3 cm internal diameter (working volume–64 ml, beads–50 g wet weight with 4 g callus). [a] Different strengths of medium like full, 1/2 and 1/4 with 20 g sucrose l^{-1} passed at different flow rates (0.3 ml min^{-1}, 0.5 ml min^{-1} and 1.0 ml min^{-1}).

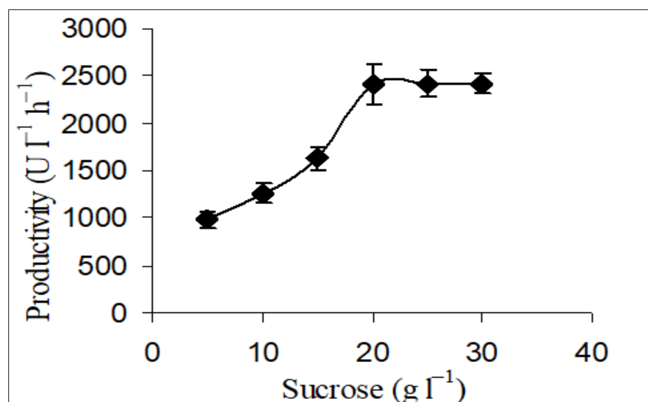

Fig 3: Change in productivity in the PBR with different concentrations of sucrose

(Half-MS, flow rate–0.3 ml min^{-1}, pH 5 and 50° C)

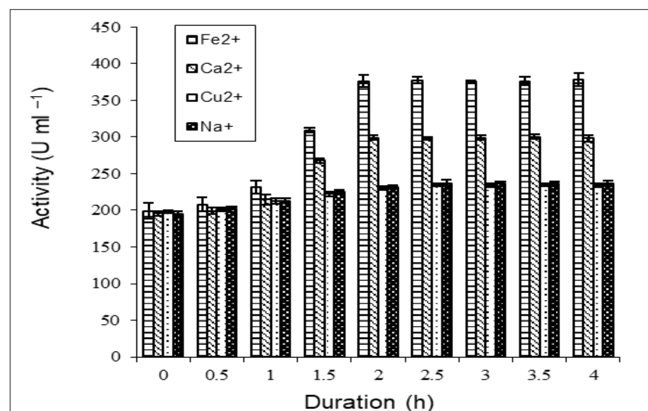

Fig 4: Effect of metal ionic concentration on the enzyme production in the reactor

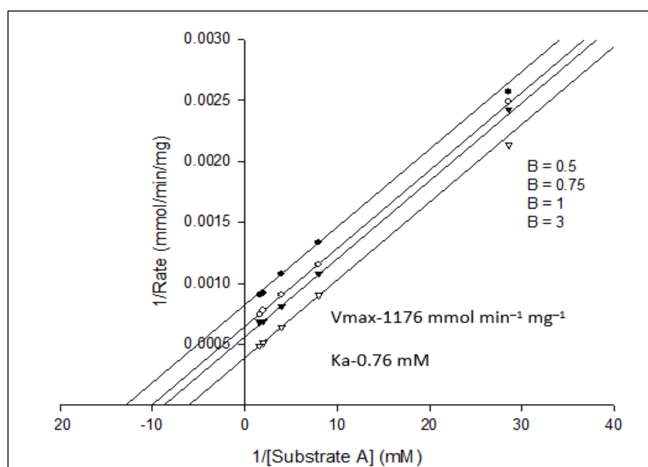

Fig 5: Lineweaver-Burk plot (Sigma plot) of substrate kinetics for the batch cultures of *Tecoma Stan* var. angustata

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